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Multifaceted transcriptional regulation of the murine intestinal mucus layer by endogenous microbiota ☆

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Abstract

The intestinal mucus layer and endogenous microbiota are strongly intertwined and this contributes to the maintenance of the epithelial barrier and ultimately of gut homeostasis. To understand the molecular foundations of such relationship, we investigated if the nature of the microbiota transcriptionally regulates mucus layer composition in vivo. We found that the expression of mucins 1 to 4 and trefoil factor 3 was down-regulated in the ileum and colon of conventional and reconventionalized mice compared with germ-free animals. Conversely, very limited colon-restricted changes in transmembrane mucins were detected in mice colonized with human adult or baby microbiota. Moreover, by microarray analysis, the murine endogenous microbiota was found to modulate genes putatively involved in mucin secretion. These findings show that a well-established microbial community participates in the regulation of the gut mucus layer and that its composition and adequacy to the host are key factors in this process.

Keywords: Bacteria; Cytoskeleton; Intestine; Microarray analysis; Mucin

It has long been alleged that the developmental program in the gastrointestinal tract is genetically pre-encoded and that genes are therefore switched on at predefined time points during the maturation process. Recently, a new hypothesis has emerged suggesting that the intestinal microbiota act as an environmental factor that drives gene expression in the host epithelium by signaling to genetically predisposed cells [1]. This hypothesis confers additional significance to the previous discovery that the endogenous microbiota directly modulate gene expression in the epithelium, thus affecting several physiological processes of the intestinal tract [2,3].

The intestinal microbial population comprises millions of cells from the Archaea, Bacteria, and Eukarya, both in mouse and in human [4]. The shaping of this endogenous community follows a typical postnatal developmental pattern, characterized by abrupt changes at weaning [5], that parallels the establishment of the mucus layer and the overall gut barrier [6]. It is straightforward to suppose that mucus and microbiota intertwine so as to impact on the maintenance of gut homeostasis and a global healthy status of the body.

The role of the microbiota in regulating the formation of a mature mucus layer may be crucial in early life and deserves more investigation. Pioneering work showed that in germ-free mice, the pre-epithelial mucus layer is reduced by half, is less stable and compact [7], and has an altered glycosylation profile compared to conventional animals [8,9]. Twelve mucin gene transcripts (MUC) have been detected in the human gastrointestinal tract [10]. MUC genes are differently expressed by all intestinal epithelium cell types, MUC1–4 being predominant. In particular, MUC2 is the main secreted colonic mucin [11]. Mucins are cosecreted with the trefoil factors [12], among which TFF3 is the most abundant in the intestine, which contribute to the viscoelastic properties of the mucus gel [13]. While gram-positive and gram-negative bacteria have been reported to up-regulate mucin expression in airway

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epithelial cells [14], in particular in case of infection [15], evidence that intestinal mucin expression may be regulated by bacteria came from studies using probiotics. In vitro, different Lactobacilli strains have been reported to either up-regulate MUC gene expression or not have any effect in colonic epithelial cell lines [16–18]. In vivo, the probiotic mixture VSL#3 was not able to modulate mucin gene expression nor thickness of the murine colonic mucus layer [19], while it produced an increase in MUC1, 2, and 3 in rat colonic loops [20]. Also, feeding 1- to 14-day-old chicks with probiotics or an antibiotic growth promoter enhanced mucin gene expression and synthesis in their jejunum and ileum [21]. Thus, the effect of nonpathogenic exogenous bacteria on intestinal mucins is still unclear. Furthermore, the effect of the endogenous bacteria is unknown.

The aim of the present study was to investigate whether bacteria residing in the gut can act as an environmental factor regulating mucus components transcriptionally. To this end, we evaluated gene expression profiles in conventional and reconventionalized mice and in mice associated with a microbiota derived from either human baby or human adult intestine and compared them to those found in germ-free animals. The association with foreign but physiologically appropriate microbiota will show if the host response is merely related to the presence of the bacteria or depends on distinct signals elicited by microbial communities of different natures.

Results

Gene expression of MUC1, 2, 3, and 4 and TFF3 in the intestine depends on the presence and composition of the gut microbiota

To investigate if the endogenous microbiota of the gut modulates, in vivo, the transcriptional expression of the major components of the mucus layer, we studied, by real-time PCR, the expression of *Muc1, Muc2, Muc3, Muc4,* and *Tff3* in the ileum and colon of conventional and germ-free mice. As shown in Fig. 1, all genes considered were found to be significantly less expressed in conventional than in germ-free animals. To tackle the effect of the nature of the intestinal microbiota on the expression of these genes, we associated germ-free mice with complex microbiota originating from murine adult, human adult, or human baby feces. Similar to conventional animals, in mice reconventionalized with murine microbiota, mucin and *Tff3* gene expression was significantly lower than in germ-free animals, in both ileum and colon. Moreover, fold changes were of analogous magnitude in conventional mice and in mice associated with murine microbiota.

Comparing gene expression in germ-free mice and in mice associated with human microbiota we observed that, in contrast to the murine community, neither type of human microbiota transcriptionally modulated *Muc2* and *Tff3* (Fig. 1). Furthermore, even though *Muc1, Muc3,* and *Muc4* were modulated by the human adult microbiota (HAM), the effect was observed only in colon and the changes were much less important than those induced by the murine microbiota. Comparing the two human bacterial communities from a global perspective, human adult microbiota induced more marked changes on MUC gene expression than human baby microbiota (HBM); however, except for *Muc4,* these variations were minor if compared to those elicited in mice reconventionalized with murine microbiota.

The microbiota regulate the expression of cytoskeleton genes putatively involved in mucus-containing vesicle intracellular trafficking

To examine if the impact of the commensal microbiota on mucus layer composition extends beyond MUC gene expression modulation, we analyzed global gene expression in the

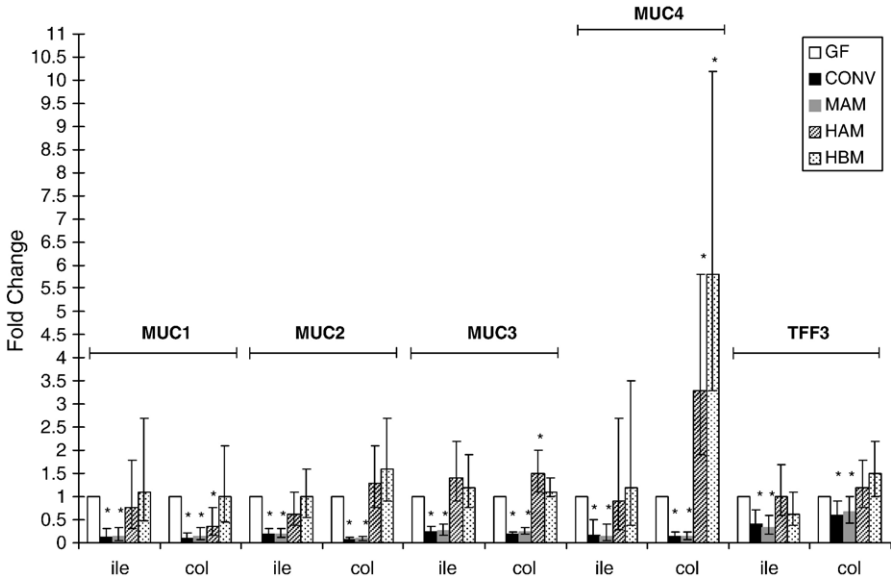


Fig. 1. Real-time PCR analysis of the expression of *Muc1–4* and *Tff3* in the ileum and colon of conventional mice (CONV) and of mice associated with fecal murine adult (MAM), human adult (HAM), or human baby (HBM) microbiota. Fold change and statistical significance were calculated versus germ-free mice after normalization to GAPDH and represent the means of five animals. Values higher than 1 indicate higher expression in colonized mice, while values between 0 and 1 indicate higher expression in germ-free mice (*significant change, test rejection level: 0.05).

Table 1
Cellular components gene ontology analysis of the probe sets modulated by the presence of the microbiota in ileum and colon at $p \leq 0.001$

GO node	Description	p value		Number of probe sets (total, up, down)	
		Ileum	Colon	Ileum	Colon
5615	Extracellular space	5.89×10^{-51}	7.63×10^{-71}	45, 14, 31	66, 30, 36
5576	Extracellular	6.89×10^{-23}	2.51×10^{-23}	10, 0, 10	8, 1, 7
5634	Nucleus	3.97×10^{-5}	3.76×10^{-16}	21, 7, 14	66, 25, 41
5768	Endosome	2.60×10^{-7}	2.27×10^{-10}	0	3, 0, 3
5770	Late endosome	1.50×10^{-9}	3.42×10^{-8}	4, 3, 1	3, 1, 2
5856	Cytoskeleton	4.16×10^{-5}	2.29×10^{-8}	2, 1, 1	3, 2, 1
5694	Chromosome	1.52×10^{-8}	2.19×10^{-4}	2, 0, 2	4
786	Nucleosome	8.59×10^{-7}	7.32×10^{-6}	1, 0, 1	4, 3, 1
5862	Muscle thin filament tropomyosin	1.92×10^{-5}	2.62×10^{-7}	1, 0, 1	2, 0, 2
5886	Plasma membrane	7.86×10^{-6}	3.34×10^{-7}	6, 6, 0	6, 3, 3
5771	Multivesicular body	1.48×10^{-6}	7.48×10^{-4}	10, 10, 0	6, 6, 0
785	Chromatin	1.49×10^{-6}	5.57×10^{-6}	1, 0, 1	2, 2, 0
15629	Actin cytoskeleton	6.39×10^{-6}	1.61×10^{-6}	3, 0, 3	6, 1, 5
5578	Extracellular matrix	2.21×10^{-6}	1.64×10^{-4}	3, 1, 2	4, 1, 3
5794	Golgi apparatus	NS	2.42×10^{-6}		5, 2, 3
5876	Spindle microtubule	4.46×10^{-6}	NS	1, 0, 1	
30027	Lamellipodium	1.22×10^{-3}	3.03×10^{-6}	2, 1, 1	3, 0, 3
42571	Immunoglobulin complex, circulating	4.81×10^{-6}	1.69×10^{-5}	6, 6, 0	5, 5, 0
19814	Immunoglobulin complex	4.81×10^{-6}	1.69×10^{-5}	0	0
5865	Striated muscle thin filament	1.48×10^{-4}	5.57×10^{-6}	0	0
48471	Perinuclear region	NS	5.57×10^{-6}		3, 0, 3
5769	Early endosome	9.92×10^{-6}	6.42×10^{-5}	2, 1, 1	2, 1, 1
9986	Cell surface	1.07×10^{-5}	NS	6, 5, 1	
119	Mediator complex	1.28×10^{-5}	3.10×10^{-3}	1, 0, 1	1, 0, 1
16324	Apical plasma membrane	1.40×10^{-5}	9.05×10^{-5}	2, 2, 0	2, 0, 2
30017	Sarcomere	6.73×10^{-5}	1.49×10^{-5}	0	0
922	Spindle pole	NS	1.72×10^{-5}		1, 0, 1
5829	Cytosol	1.86×10^{-5}	1.03×10^{-4}	6, 3, 3	7, 5, 2
30016	Myofibril	7.71×10^{-5}	1.78×10^{-5}	0	0
5813	Centrosome	NS	1.82×10^{-5}		2, 0, 2
5819	Spindle	1.11×10^{-3}	4.31×10^{-5}	0	0
30484	Muscle fiber	1.51×10^{-3}	7.15×10^{-5}	0	1, 1, 0
9897	External side of plasma membrane	8.50×10^{-5}	NS	8, 8, 0	
5815	Microtubule organizing center	NS	2.68×10^{-4}		0
5581	Collagen	3.38×10^{-4}	NS	2, 0, 2	
19815	B cell receptor complex	4.44×10^{-4}	8.33×10^{-4}	6, 6, 0	5, 5, 0
1772	Immunological synapse	4.72×10^{-4}	NS	0	
12506	Vesicle membrane	4.04×10^{-3}	7.72×10^{-4}	1, 0, 1	1, 0, 1
5942	Phosphoinositide 3-kinase complex	1.44×10^{-3}	4.91×10^{-3}	1, 0, 1	1, 0, 1
5730	Nucleolus	1.69×10^{-3}	NS	2, 0, 2	
12505	Endomembrane system	NS	1.86×10^{-3}		0
5884	Actin filament	1.88×10^{-3}	NS	2, 0, 2	
5764	Lysosome	NS	1.99×10^{-3}		4, 3, 1
15630	Microtubule cytoskeleton	NS	2.28×10^{-3}		2, 2, 0
5657	Replication fork	2.32×10^{-3}	NS	1, 0, 1	
5654	Nucleoplasm	NS	2.50×10^{-3}		0
16604	Nuclear body	NS	2.73×10^{-3}		1, 0, 1
5777	Peroxisome	NS	2.79×10^{-3}		3, 1, 2
5789	Endoplasmic reticulum membrane	NS	2.87×10^{-3}		2, 2, 0
16591	DNA-directed RNA polymerase II, holoenzyme	3.13×10^{-3}	NS	0	
42175	Nuclear envelope–endoplasmic reticulum network	NS	3.14×10^{-3}		0
5956	Protein kinase CK2 complex	NS	3.22×10^{-3}		1, 1, 0
1750	Photoreceptor outer segment	NS	3.33×10^{-3}		1, 1, 0
42579	Microbody	NS	3.43×10^{-3}		0
16323	Basolateral plasma membrane	NS	3.75×10^{-3}		1, 1, 0
45095	Keratin filament	NS	3.82×10^{-3}		1, 1, 0
323	Lytic vacuole	NS	4.01×10^{-3}		0
30891	VCB complex	NS	6.65×10^{-3}		1, 0, 1
776	Kinetochore	NS	6.92×10^{-3}		2, 0, 2
42589	Zymogen granule membrane	NS	7.48×10^{-3}		1, 1, 0
9925	Basal plasma membrane	NS	7.48×10^{-3}		1, 1, 0

Table 1 (*continued*)

GO node	Description	*p* value		Number of probe sets (total, up, down)	
		Ileum	Colon	Ileum	Colon
5773	Vacuole	NS	8.22×10^{-3}		0
42613	MHC class II protein complex	8.84×10^{-3}	NS	1, 1, 0	
42611	MHC protein complex	8.84×10^{-3}	NS	0	
30667	Secretory granule membrane	NS	9.96×10^{-3}		0
42588	Zymogen granule	NS	9.96×10^{-3}		0
5912	Adherens junction	NS	9.98×10^{-3}	0	1, 0, 1

Only nodes associated with a $p \leq 0.01$ are listed (GO, Gene Ontology; NS, not significant). In the last two columns, up and down indicate how many probe sets, among the total contained in each node, had a higher or lower expression in conventional mice, respectively; annotation of each probe set and corresponding fold are given in Supplementary Table S1. "0" indicates that probe sets annotated to the node could be further associated with its children (more specific) and therefore they do not appear in the parent node; they can be found in Supplementary Table S2.

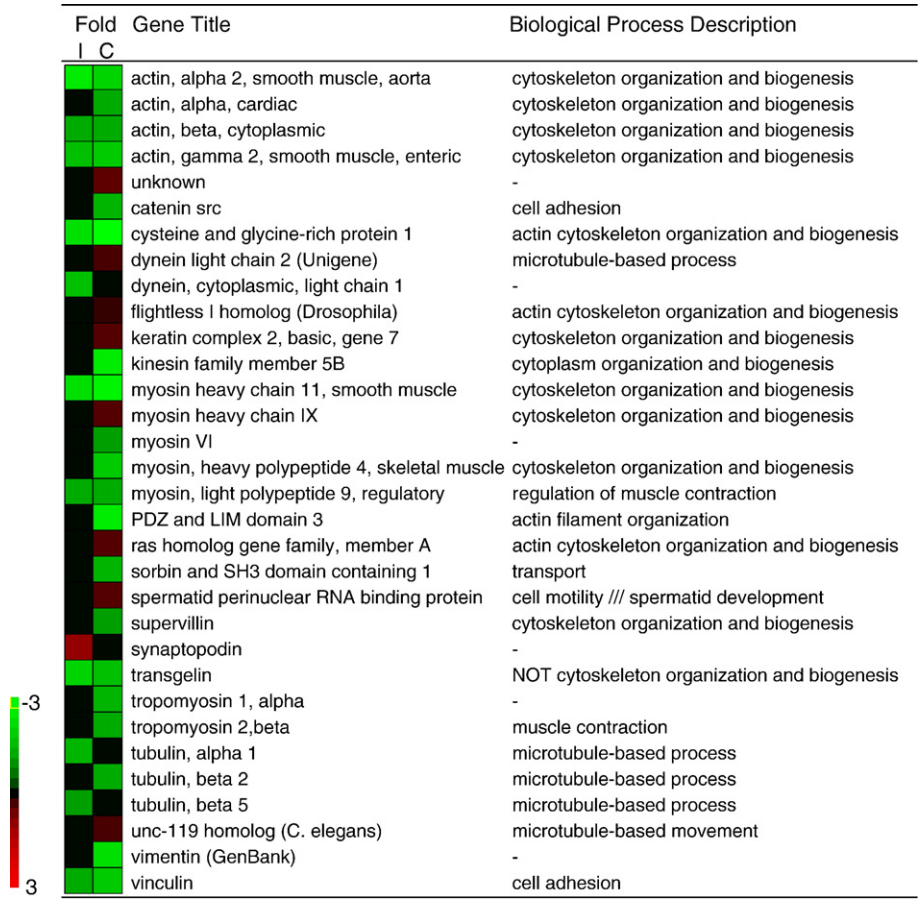
ileum and colon of germ-free and conventional animals by microarray technology. Twelve chips were used (6 per group). The presence of the microbiota significantly affected 739 and 1600 probe sets in ileum and colon, respectively ($p \leq 0.001$). A complete list of these probe sets, together with their annotation and associated signal intensities, is given in Supplementary Table S1. While *Muc2* and *Muc4* are not included in the MGU74Av2 array, probe sets representing *Muc1, Muc3,* and *Tff3* do not appear in this list, since they were not selected by the statistical analysis as being differentially expressed. This may be due to intrinsic limits of the Robust Multiarray Analysis algorithm when applied to probe sets associated with low expression or small fold change [22,23].

We analyzed modulated genes using the biological processes, molecular function, and cellular components gene ontologies, as described under Materials and methods. The entire set of data is presented in Supplementary Table S2. In the three ontologies, both nodes commonly or specifically modulated in ileum and/or colon could be identified. Among the biological processes, the glucocorticoid receptor signaling pathway (Gene Ontology (GO) ID 42921) and regulation of transport (GO ID 51049) were those associated with the lowest *p* value in ileum and colon, respectively, but a number of processes were commonly regulated in both regions, including intracellular signaling cascade (GO ID 7242), B cell activation (GO ID 42113), and chromatin assembly or disassembly (GO ID 6333). Clustering with the molecular function ontology revealed protein binding (GO ID 5515) as the node with the most significant regulation, in both ileum and colon. Several functions were specifically modulated in each tissue, including ligand-dependent nuclear receptor transcription coactivator activity (GO ID 30374) and RNA polymerase II transcription mediator activity (GO ID 16455) in the ileum and zinc ion binding (GO ID 8270) and chromatin binding (GO ID 3682) in the colon, while others such as cytoskeletal protein binding (GO ID 8092) were modulated in both regions. Finally, according to the cellular component ontology analysis the extracellular space node (GO ID) was found to be the most modulated in ileum and colon. Most of the other nodes, such as endosome (GO ID 5768), nucleus (GO ID 5634), cytoskeleton (GO ID 5856), and chromosome (GO ID 5694), among the top ones, were significantly modulated in both regions (Table 1).

Even though a node was found to be commonly modulated in ileum and colon, the number of genes mapping to the node could be different between the two regions. Moreover, there might be a different number of up- and down-regulated probe sets associated with it. This is exemplified in Table 1 for the cellular component ontology analysis and may indicate a region-specific effect of the gut microbiota.

As noted above, the cytoskeleton node was among those commonly modulated in ileum and colon. Cytoskeleton components, namely microtubules and actin filaments, have been previously described to be involved in the secretion of mucin granules in the intestine [24,25]. Therefore, we hypothesized that mucin trafficking and secretion might be affected by the microbiota. To extrapolate relevant data, we constructed a gene set containing genes coding for proteins known to be part of or to be involved in the synthesis of the cytoskeleton, or to interact with any of its components, and we examined their modulation in our Affymetrix dataset. Six and sixteen percent of the probe sets contained in this gene set were significantly modulated by the microbiota in the ileum and colon, respectively. Moreover, in the two intestinal regions, most of the genes being regulated were less expressed in conventional than in germ-free animals (Fig. 2). By clustering the differentially expressed genes using gene ontologies, the cytoskeleton organization and biogenesis node were found to be significantly modulated in both tissues ($p \leq 0.001$). The expression of genes coding for the three main components of the eukaryote cytoskeleton, namely microfilaments (actin), intermediate filaments (keratin), and microtubules (tubulin), were affected by the microbiota (Fig. 2). In particular, cytoplasmic β-actin was more highly expressed in germ-free mice. An increased synthesis of actin has been correlated with less secretion of mucin granules from airway goblet cells [26]. Our data point to the fact that this may be true in the intestine as well.

Furthermore, we considered the possible involvement of the microbiota in modulating mucin glycosylation pattern. Indeed, while mucin glycan chains may mediate attachment as well as serving as a carbon source for commensal bacteria [27], it is still unclear to what extent the microbial community affects their synthesis. Mucin glycan synthesis starts with the addition of *N*-acetylgalactosamine to serine or threonine residues of the apomucin. The glycan is then sequentially elongated by the addition of galactose and/or *N*-acetylglucosamine moieties, frequently



Fold I	Fold C	Gene Title	Biological Process Description
		actin, alpha 2, smooth muscle, aorta	cytoskeleton organization and biogenesis
		actin, alpha, cardiac	cytoskeleton organization and biogenesis
		actin, beta, cytoplasmic	cytoskeleton organization and biogenesis
		actin, gamma 2, smooth muscle, enteric	cytoskeleton organization and biogenesis
		unknown	-
		catenin src	cell adhesion
		cysteine and glycine-rich protein 1	actin cytoskeleton organization and biogenesis
		dynein light chain 2 (Unigene)	microtubule-based process
		dynein, cytoplasmic, light chain 1	-
		flightless I homolog (Drosophila)	actin cytoskeleton organization and biogenesis
		keratin complex 2, basic, gene 7	cytoskeleton organization and biogenesis
		kinesin family member 5B	cytoplasm organization and biogenesis
		myosin heavy chain 11, smooth muscle	cytoskeleton organization and biogenesis
		myosin heavy chain IX	cytoskeleton organization and biogenesis
		myosin VI	-
		myosin, heavy polypeptide 4, skeletal muscle	cytoskeleton organization and biogenesis
		myosin, light polypeptide 9, regulatory	regulation of muscle contraction
		PDZ and LIM domain 3	actin filament organization
		ras homolog gene family, member A	actin cytoskeleton organization and biogenesis
		sorbin and SH3 domain containing 1	transport
		spermatid perinuclear RNA binding protein	cell motility /// spermatid development
		supervillin	cytoskeleton organization and biogenesis
		synaptopodin	-
		transgelin	NOT cytoskeleton organization and biogenesis
		tropomyosin 1, alpha	-
		tropomyosin 2,beta	muscle contraction
		tubulin, alpha 1	microtubule-based process
		tubulin, beta 2	microtubule-based process
		tubulin, beta 5	microtubule-based process
		unc-119 homolog (C. elegans)	microtubule-based movement
		vimentin (GenBank)	-
		vinculin	cell adhesion

Fig. 2. Genes contained in the Cytoskeleton gene set and associated with a fold change higher than 1.5 ($p \leq 0.001$) and with an expression level of at least 150 in at least one intestinal region. Gene titles correspond to the Affymetrix annotation, unless indicated in parentheses. Biological process descriptions were obtained by clustering differentially expressed genes using Gene Ontology and correspond to the deepest significant nodes ($p < 0.001$). Fold change represents the ratio of expression in conventional versus germ-free mice (I, ileum; C, colon).

modified by sulfate groups, and finally terminated by sialic acid or fucose [28]. We constructed a gene set containing probe sets representing glycosyltransferases and nucleotide sugar transporters. Since not all of these genes have been assigned to GO nodes yet, we used the glycogene list compiled from the Consortium for Functional Glycomics (www.functionalglycomics.org) for category assignments. The expression of most of the glycogenes was not affected by the presence of the microbiota. Nevertheless, in this case as well, the number of probe sets significantly modulated in colon exceeded the number of those modulated in ileum. Moreover, except for ST6GalNAc4 (96682_at) and ppGalNAcT4 (101333_at), whose regulation (up and down, respectively) is borderline in the colon of conventional animals, among the glycogenes represented on the MG-U75Av2 chip, those modulated by the microbiota are mostly involved in the synthesis of N-linked glycans (94432_at, 101074_at, 95417_at, 99423_at), glycolipids (102936_at, 100369_at, 98872_at, 94197_a), and proteoglycans (102811_at, 102410_at) and not in mucin glycosylation (O-glycans) (Supplementary Table S1).

Discussion

The involvement of the endogenous microbiota in the regulation of the intestinal epithelial barrier function is predictable but the molecular mechanisms remain largely unknown. In the present study, we show that the intestinal microbiota transcriptionally regulates the mucus layer and that its bacterial composition influences this process. First, we compared conventional and germ-free mice to establish the involvement of gut microbiota on mucin expression. We observed that, while all of the MUC genes considered are expressed in both conventional and germ-free animals, they all are down-regulated in the first. This result is consistent with previous findings documenting increased mucin protein synthesis in germ-free mice [29], despite the reduced number of goblet cells in their intestinal epithelium [30,31]. These data, combined with the observation of higher trefoil factor 3 expression in germ-free animals, suggest that these mice may up-regulate mucin synthesis as a defense mechanism, so as to compensate for the lack of endogenous microbiota acting as a first defense against luminal aggression.

To investigate further the impact of the bacterial ecosystem as an environmental factor affecting mucin gene expression, we used mice associated with microbiota of different compositions and origins. Gene modulation in animals associated with murine adult microbiota was analogous to that observed in conventional mice, showing that the reconventionalization procedure does not impact on the measured outcome. In contrast to murine adult microbiota, human microbiota did not dramatically affect

mucin gene expression. Even if the human adult microbiota induced some changes in MUC expression in the colon, the magnitude of these changes was low compared to those induced by the murine microbiota. In addition, human baby microbiota had even less impact than that of the human adult, suggesting that some bacterial groups, absent from the baby microbiota, are likely required to produce adequate signals. These findings show that the cross talk between bacteria coming from a human ecosystem and the mouse intestine is not as efficient as in conventional or reconventionalized mice and highlight that the adequacy between the host and its own bacterial profile is likely to play a key role in modulating mucus composition. This hypothesis is further supported by the fact that MUC4, a membrane mucin implicated in cellular signaling [32], is affected by the human and murine microbiota in opposite manners.

Moreover, previous studies of germ-free mice associated with a human adult microbiota showed that, in these animals, the development of the small intestinal immune system is impaired compared to in conventional mice, due to the lack of colonization by segmented filamentous bacteria [33,34]. In the present study, we observed a good establishment of Enterobacteriaceae, Bacteroides, and Bifidobacteria in the murine gut, independent of the age of the human donor (Supplementary Table S3). In contrast, human Enterococci and Lactobacilli did not colonize the intestine of HAM mice and were absent in the gavage administered to HBM mice. This suggests that these groups of bacteria may play a role in the regulation of mucin expression in the intestinal tract. Previously published studies show that, in vitro, probiotic Lactobacilli strains such as *Lactobacillus rhamnosus* GG and *Lactobacillus* Lp299v can up-regulate colonic expression of MUC2 and MUC3 [16,35], as well as, in vivo, the probiotic mixture VSL#3, which contains *Streptococcus, Lactobacillus,* and *Bifidobacterium* species [20]. On the other hand, *Lactobacillus salivarius* UCC118 and *Lactobacillus acidophilus* DDS did not increase MUC3 expression in the HT-29 colonic epithelium cell line [16,18]. Therefore MUC gene expression regulation by Lactobacilli seems to be strain-specific. Our results suggest that a complex bacterial community containing Lactobacilli would down-regulate MUC gene expression, in both ileum and colon. However, neither previous results nor ours permit a conclusion on the involvement of the gut-resident Lactobacilli in mucin expression regulation, and more thorough experiments would be needed to investigate this matter further.

In any case, since the response of the intestinal epithelium to microbial colonization depends on the origin of the bacteria, as discussed above, to investigate further the molecular foundations of the gut endogenous bacteria–mucus relationship in a physiologically relevant system we used conventional and germ-free animals.

To find out if there are mucus-related processes transcriptionally affected by the microbiota other than MUC gene expression, we ran a whole-genome gene expression analysis and found that several cytoskeleton genes are significantly less expressed in the ileum and colon of conventional animals compared to germ-free. Cytoskeletal genes have also been reported to be modulated by the gut resident bacterium *Bacteroides thetaiotaomicron* in the murine ileum [2]. Modulated genes include members from the three constituents of the cytoskeleton, namely actin microfilaments, microtubules, and keratin intermediate filaments. These, and in particular actin, have been described to be involved in mucus granule secretion [24–26]. Therefore, these findings suggest an involvement of the microbiota in mucus granules trafficking. Microdissection of goblet cells and enterocytes and analysis of the cell-type-specific mRNA profiles would confirm this hypothesis.

Finally we evaluated the impact of the microbiota on glycogene expression. Mainly enzymes involved in the synthesis of N-glycans, and not O-glycans, appeared to be transcriptionally modulated, thus excluding an impact of the microbiota on mucin glycan chain extension.

In summary, this study suggests that the impact of the gut microbial community on the composition of the mucus layer spans different levels at the transcriptional stage. These comprise MUC gene expression regulation and possibly mucin secretion. Moreover, gut microbiota composition and its adequacy to the host are important factors in this process. Hence, interventions leading to modifications of the gut microbiota could be a strategy to modulate mucus composition in the intestinal tract and therefore to treat intestinal pathologies that affect the mucus barrier, such as mucositis associated with cancer chemo- and radiotherapy.

Materials and methods

Animals and diet

Germ-free and conventionally raised male C3H mice, 6 weeks of age, were obtained from the Nestlé Research Center Animal House (day 1). The germ-free animals were distributed into three groups ($n=5$) to be immediately associated, by intragastric gavage, with either human baby or human adult fecal microbiota suspended in PBS (0.2 ml per animal). For the protection of extremely oxygen-sensitive bacteria, the gavage suspensions were prepared in an anaerobic glove box using prereduced medium and the samples were then protected from oxygen until gavage. The same gavage was repeated on day 2. Animals belonging to the third group remained germ-free and were given a PBS gavage. Absence of bacterial colonization in these animals was verified throughout the entire study by microscopy examination of feces and by culturing fecal samples on nonselective medium in aerobiosis and anaerobiosis. Alternatively, germ-free mice were associated with murine adult fecal microbiota, following the same protocol. The animals were maintained in sterile plastic isolators, except for the conventional group ($n=5$), and were fed on a standard UAR 03-40 diet (SAFE, Villemoisson/Orge, France), sterilized by γ-irradiation, and sterile water ad libitum. The animals were sacrificed on day 22 by exhaustive bleeding under isoflurane anesthesia. The whole intestine was immediately removed and the contents of the small intestine and colon were collected for microbiota composition analysis. The tissues corresponding to the ileum and colon were dissected, snap-frozen, and stored at −80 °C for RNA preparation. The study design and procedures were approved by the Service Vétérinaire Cantonal ethical committee (Lausanne, Switzerland).

Gavages and gut microbiota composition analysis

The fecal murine microbiota gavage was prepared by 100-fold dilution in PBS of 2 g of freshly passed feces collected from adult males. The fecal human microbiota gavage was obtained by 100-fold dilution in PBS of 5 g of freshly passed feces collected from an adult male. The human baby microbiota gavage consisted of a consortium of seven species, previously isolated from the feces of a 20-day-old baby delivered naturally and breast-fed, comprising *Bifidobacterium breve* NCC452, *Bifidobacterium longum* NCC572, *Staphylococcus aureus* FSM124, *Staphylococcus epidermidis* FSM115, *Escherichia coli* FSM325, *Bacteroides distasonis* FSM24, and *Clostridium perfringens* FSM C14.

Freshly passed fecal samples collected at day 16 and intestinal contents collected at sacrifice from small intestine and colon were immediately analyzed for the endogenous populations on selective or semiselective media, as previously described [36]. Total aerobes and anaerobes were quantified on trypticase soy agar supplemented with 5% sheep blood (bioMerieux, Marcy L'Etoile, France) and *S. aureus* on Chapman medium (bioMerieux). The bacterial load of the gavage solutions, of the intestinal contents, and of feces is given in Supplementary Table S3.

RNA preparation

Total RNA was extracted from whole-thickness ileum and colon using the Tripure Isolation reagent (Roche Diagnostics AG, Rotkreuz, Switzerland) following the manufacturer's protocol and further purified with the Nucleospin kit (Macherey-Nagel AG, Oensingen, Switzerland), which includes an on-column DNase I treatment step. The recovered RNA was quantified using the RiboGreen RNA Quantification kit (Molecular Probes, Basel, Switzerland) and its quality was assessed with an Agilent 2100 Bioanalyzer and the RNA 6000 Nano LabChip kit (Agilent Biotechnologies, Waldbronn, Germany).

Real-time RT-PCR analysis of mucins and Tff3 gene expression

TaqMan real-time PCR was carried out in a ABI 5700 thermocycler (Applied Biosystems, Rotkreuz, Switzerland) using RNA reverse-transcribed with the Reverse Transcription System (Catalys AG, Zurich, Switzerland) and oligo(dT) as a primer. Primers and TaqMan probes were purchased from Applied Biosystems; those for *Muc1, Muc2, Muc4,* and *Tff3* were available on demand, while those for *Muc3* were obtained through the By Design service and were as follows: forward primer, 5′-TGGTCAACTGCGAGAATGGA-3′; reverse primer, 5′-GAGGCTGGTGCACTGACATTT-3′; TaqMan probe, 5′-6-carboxyfluorescein–ACGTGGGACGGGCT-3′. Before use, this primer set was validated and its linearity range of application determined. The reactions were carried out in triplicate. Data were analyzed using the GeneAmp 5700 SDS software (Applied Biosystems). Relative gene expression was calculated after normalization to GAPDH, whose expression had been shown not to vary across samples by preliminary experiments. Statistical significance of differential expression versus germ-free mice was determined a posteriori after an ANOVA (analysis of variance) using a General Linear model procedure. Factors were treatment, animal nested in treatment, and gene; interactions were treatment × gene and animal × gene. The mean-square interaction animal × gene term was used to calculate the significance of the changes versus germ-free mice.

Microarray hybridization

Five micrograms of total RNA obtained from whole-thickness ileum and colon of three mice per group (conventional and germ-free) was independently reverse transcribed, amplified, and biotin labeled using the SuperScript Choice System (Invitrogen AG, Basel, Switzerland) and the Bioarray High Yield RNA Transcription Labeling Kit (Enzo Life Sciences, Farmingdale, NY, USA). The obtained cRNAs were hybridized to the Murine Genome Array U74Av2 (Affymetrix, High Wycombe, UK) according to the manufacturer's protocols. The chips were scanned at 488 nm with an argon-ion laser (Agilent Biotechnologies, Rotkreuz, Germany) and expression signals were generated using the Robust Multiarray Analysis algorithm [37]. The whole dataset has been deposited at the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/) under Accession No. GSE3304.

Microarray data analysis

Statistical significance of differential gene expression between germ-free and conventional mice was evaluated by two-factors ANOVA followed by a posteriori treatment comparison in each of the two intestinal regions, using the Global Error Assessment model [38] at a test rejection level of 0.001. Genes significantly affected by the presence of the microbiota were then functionally clustered using GO (http://www.geneontology.org/) and an in-house-implemented procedure based on determining the proportion of genes mapping to each GO node, as previously described [3]. This allowed not only the functional annotation of the selected genes according to the GO database, but also the identification of significantly modulated biological processes and cellular components ($p < 0.001$). In addition, to mine for relevant data, we constructed two gene sets named Cytoskeleton and Glycogenes, containing 511 and 125 probe sets, respectively, using as information sources the literature and gene ontologies. Since not all of the glycogenes have been assigned to GO nodes yet, for this gene set we used the glycogene list compiled by the Consortium for Functional Glycomics (www.functionalglycomics.org). The list of probe sets included in each gene set is provided in Supplementary Table S4. A fold of 1.4 has been suggested as the lowest that can be accurately detected [39] and has indeed been used in studies employing the same chip as the current research [40]. Therefore, to identify biological processes significantly modulated by the endogenous microbiota within the gene sets, genes associated with an expression signal of at least 150 and a significant fold change between conventional and germ-free mice of at least 1.5 were selected to be clustered using the Biological Process Gene Ontology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2007.09.006.

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